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Essential arterial hypertension and polymorphism of angiotensinogen M235T gene

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Abstract

Several candidate genes, chosen from the renin- angiotensin system, were examined for their association with essential hypertension. The genes of the renin- angiotensin system (RAS) are good candidates for such an approach because this system is well known to be involved in the control of blood pressure. One of these candidate genes is the gene encoding for angiotensinogen (the most important gene of the RAS associated with essential hypertension in the most population, is the gene for angiotensin-converting enzyme- ACE). One DNA polymorphism within exon 2- with threonine instead of methionine at position 235 (M235T) was found to be significantly associated with hypertension. The objective of this study is the analysis of M235T polymorphism in angiotensinogen gene in Romanian patients with essential hypertension as well as controls. We examined 38 patients with essential hypertension and 21 normotensive patients. In order to identify the M235T angioteninogen variant, we used the following methods: DNA extraction, PCR amplification and enzymatic digestion of the PCR product using *Tth 111I* restriction endonuclease enzyme. In the study groups, the M235T variant (Met?Thr in aminoacid position 235) was found more frequently in hypertensive patients (81,57%), than in control subjects (66,66%). We identified 52,63% M235T heterozygotes in the hypertensive group compared with 47,61% in the control group, and 28,94% T235T homozygotes in the hypertensive group compared with 19,04% in the control group. The results of our study suggest an association of the M235T polymorphism in the gene encoding angiotensinogen with essential hypertension.

Keywords: essential hypertension - angiotensinogen gene polymorphisms - M235T variant - polymerase chain reaction - Romania

Introduction

Human hypertension has been recognized as one of the most important risk factors for the development of cardiovascular diseases such as coronary heart disease (CHD), myocardial infarction (MI) or stroke, all of which are principal causes of human cardiovascular morbidity and mortality [1, 2].

Despite hypertension's high prevalence and great impact on public health, little is still known about its causes. In the majority of patients with hypertension, no anatomic, metabolic, or endocrine derangement can be found, and hypertension in these cases is regarded as a primary or essential hypertension. At present it is widely accepted that

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approximately 30-50% of cases of hypertension can arise from genetic susceptibility [3]. This view is strongly suported by twin studies that demonstrate familial aggregation of the disease [3]. Human hypertension is a complex, polygenic disease in which one or more gene control the level of blood pressure [1, 4].

Several candidate genes, all chosen from the renin- angiotensin system, have been examined. One of them is the gene encoding for angiotensinogen, precursor of the vasoactive peptide angiotensin II [5,6]. The most important gene of the RAS associated with essential hypertension in the most population, is the gene for angiotensin-conversing enzyme- ACE.

Angiotensinogen , the renin substrate, is mainly synthesized by the liver under the positive control of estrogens, glucocorticoids, thyroid hormones and angiotensin II. Angiotensinogen is the unique substrate for renin [1]. The plasma angiotensinogen level reflects mainly this synthesis. The brain, large arteries, heart, kidney, and adipose tissues are also sites of angiotensinogen synthesis. The mature form consists of 452 amino acid residues [7]. The human angiotensinogen gene has been localized on chromosome 1q42-3 and encompasses five exons and four introns spanning 13Kb of genomic sequence [8]. Different studies have indicated a relationship between plasma angiotensinogen level and blood pressure in humans [9]. Higher level of angiotensinogen have been observed in hypertensive subjects than in normotensive patients [10,11]. Several polymorphisms in the AGT gene have been identified [12]. One DNA polymorphism within exon 2- with threonine instead of methionine at position 235 (M235T) was found to be significantly associated with hypertension (1,4). A significant increase in plasma angiotensinogen level was observed for patients carrying the 235T variant, with or 20 percent increase in heterozygotes (MT) and homozygotes (TT) respectively, compared with wild- type homozygotes (MM) [13]. These results were confirmed in hypertensive patients regardless of family history of hypertension [13]. The results of all these studies favor the hypothesis that angiotensinogen is a determinant of blood pressure.

The aim of this study is: the analysis of M235T polymorphism in the angiotensinogen gene in Romanian patients with essential hypertension as well as controls. This is the first such study carried out in Romania, initiated to investigate the role of genetic factors in the pathogenesis of hypertension. We will try to compare our results with those from other studies carried out in different populations.

Materials and methods

Hypertensive patients

The study group consisted of 38 patients with essential hypertension. All patients were hospitalised in the Department of Cardiovascular Disease. The study group consisted of 15 males (39,47%) with the mean age of 55±8,4 years and 23 females (60,52%) with the mean age of 58±10,86 years.

Risk factors for essential hypertension were obtained by standard questionnaire, physical examination and blood tests. The patients were asked about their personal medical history, family history of hypertension, smoking habit, alcohol consumption. Blood pressure was measured by standard methods. All these patients had hypertension onset before the age of 60 years. We considered that patients had essential hypertension if they had had diastolic blood pressure (BP) above 95mm Hg and systolic BP above 140mm Hg at the time of examination and they had no clinical evidence of secondary hypertension. Positive history of hypertension was reported by 24 patients (63,15%). Twenty patients (52,63%) had positive familial history of hypertension. None of these patients had diabetes.

Control group

The control group consisted of 21 age- matched individuals. The group of normotensive individuals included 9 men (42,85%) and 12 (57,14%) women. The mean age of the normotensive women was 53,16± 12,26 years and the mean age of the normotensive men was 51,55±20,89 years. None of the individuals from the control group had symptoms of essential hypertension. They had systolic BP lower than 140mm Hg, and diastolic BP lower than 90mm Hg. None of the control group subjects had positive familial history of essential hypertension or myocardial infarction (MI). None of these individuals had secondary hypertension or diabetes. All the individuals from the control group had never been treated with anti- hypertensive medication.

All patients agreed to take part of this study and they respond at one questionnaire to provide information about hypertension risk factors such as: cigarettes smoking, alcohol consumption, familial history of hypertension. On the other hand, the study had the agreement of the ethical board for the DNA analysis.

The main charactheristics of both groups are presented in Table 1.

Blood pressure measurements

Blood pressure was measured using a mercury-gravity manometer. Measurements were taken at the left arm with subjects in the seated position after 10 minutes of resting. This procedures was repeated three times, with systolic and diastolic blood pressures defined to be the mean of the three independent measurements.

DNA isolation and polymerase chain reaction

DNA extraction was performed at the Department of Medical Biochemistry of University of Medicine and Pharmacy Cluj.

In order to identify M235T mutation 5ml of venous blood were drawn into EDTA tubes containing 100µl of 15% EDTA. DNA was isolated from peripheral blood leukocytes using the method of Lahiry [14]. After the lysis of red blood cells and the precipitation and elimination of proteins, DNA was precipited with ethanol and redissolved, depending on the pellet size, in 250-500µl TE (1mM EDTA, 10mM Tris-HCl) [14]. DNA concentration was from the optical density at 260nm. All DNA samples were pure.

The polymerase chain reaction (PCR) was used to examine M235T angiotensinogen polymorphism. In order to amplify the region encompassing point mutation M235T, we used the primers described by Russ and Niu [15, 16].The primers have the following sequences: the forward primer:

5'-CAGGGTGCTGTCCACACTGGACCCC-3' and the reverse primer:

5'-CCGTTTGTGCAGGGCCTGGCTCTCT-3' [15,16].

The volume of each PCR reaction was 100ìl and contained: 400ng genomic DNA, 0.1µM primers (Sigma Genosys), 200µM of each deoxynucleotide triphosphates (dNTP), 2.0mM $MgCl_2$ and 2 units of Taq DNA polymerase (Sigma). The reaction was performed in Eppendorf themocycler using the following program of amplification: initial denaturation of DNA duplex 10 minutes at 95ºC, followed by 35 cycles of: 1 minute of denaturation at 94ºC, 1 minute of primers annealing at 59ºC and 1 minute and 30 seconds of primers extension at 72ºC. Final elongation was for 10 minutes at 72ºC.

Digestion of PCR products with restriction endonuclease enzyme (restriction fragment length polymorphism- RFLP)

The M235T gene polymorphism was detected by digestion of the PCR product with restriction endonuclease enzyme *Tth111*I (New England Biolabs). This technic called restriction fragment length polymorphism - RFLP is one of the most important technics used in molecular biology to identify DNA point mutation. The digestion was performed in 10µl reaction mixture and contained: 1U *Tth111*I/ 3µl PCR product. The restriction mixture was digested for 3h at 65ºC in the buffer supplied by manufacturer (New England Biolabs). DNA fragments were visualized in 12% polyacrilamide gel stained with 8µl of 10mg/ml ethidium bromide. For the normal (wild type) genotype (MM) the PCR product is of 165bp, with no cutting site for *Tth111I*. Subjects homozygous for M235T (TT genotype) have after enzymatic digestion with *Tth111I*

Table 1. Characteristics of patients from the two study groups.

Characteristics of patients	Hypertensive (n=38)	Normotensive (n=21)
Women nr (%)	23 (60,52%)	12 (57,14%)
Mean age (years)	58 +/- 10,86	53,16 +/- 12,26
Alcohol consumption nr (%)	2 (8,69%)	2 (16,66%)
Cigarette smoking (at least 10 cigarettes daily)	2 (8,69%)	1 (8,33%)
Familial history of hypertension	8 (34,78%)	0 (0%)
Men nr (%)	15 (39,47%)	9 (42,85%)
Mean age (years)	55 8,4	51,55 20,89
Alcohol consumption nr (%)	3 (20%)	5 (55,55%)
Cigarette smoking (at least 10 cigarettes daily)	5 (33,33%)	2 (22,22%)
Familial history of hypertension	12 (80%)	0 (0%)

Table 2. Distribution of the M235T angiotensinogen variant among the hypertensive and normotensive patients.

Genotype	Hypertensive	Normotensive
Total	38	21
TT genotype no (%)	11 (28,94%)	4 (19,04%)
MT genotype no (%)	20 (52,63%)	10 (47,61%)
MM genotype no (%)	7(18,42%)	7(33,33%)

- TT genotype- patients homozygous for M235T angiotensinogen variant with two copies of the T235 allele
- TM genotype- patients heterozygous for M235T angiotensinogen variant with one copie of the T235 allele
- MM genotype- patients negative for M235T angiotensinogen variant with two copies of M235 allele.

two fragments of 141 and 24bp. Subjects heterozygotes for M235T (TM genotype) have two fragments of 165, 141 and 24bp (15,16) (Fig. 1). In polyacrilamide gel we can see only the 165 and 141bp fragments.

Results

Table 2 shows the genotype distribution for M235T polymorphism in the AGT gene.

In the study groups, the M235T variant (Met?Thr in aminoacid position 235) was found more frequently in hypertensive patients (81,57%), than in control subjects (66,66%). We also studied the frequency of the M235T variant between hypertensive women and normotensive women and respectively between hypertensive and normotensive men. We found no significant difference in genotype frequencies between hypertensive (78,26% -18/23) and normotensive women (75% -9/12). On the other hand, in men important differences in genotype frequencies were observed between hypertensive and normotensive men (86,66%- 13/15 and 55,55%-5/9, respectively). The distribution of the genotypes with T235 allele was also significantly different between the two groups of patients. Among the 38 patients with essential hypertension, 52,63% (20/38) were heterozygous (genotype TM) and 28,94% (11/38) were homozygous (genotype TT), while among 21 normotensive patients 47,61% (10/21) were heterozygous (genotype TM) and 19,04% (4/21) were homozygous (genotype TT). The frequency of TM and TT genotypes were higher in hypertensive patients than in normotensive patients.

Discussion

The genes of the renin- angiotensin system (RAS) are good candidates for the study of the genetics of HTA because this system is well known to be involved in the control of blood pressure.

In the past few years, there were many studies that confirmed a positive association between M235T variant and hypertension and on the other hand there were many studies who did not detect any association at all.

An extensive study of the potential role of the angiotensinogen gene in human essential hypertension was recently performed by Jeunemaitre *et al.* (1992) in two large series of 379 sibling pairs (135 French sibling pairs from Paris and 244 American sibling pairs from Salt Lake City, Utah) [1]. He found genetic linkage between essential hypertension and AGT in affected siblings and an elevated M235T allele frequency as compared to normotensive patients. In 1993, the same group studied 119 hypertensives from Paris, France, patients with positive familial history of hypertension. The results confirmed the association between M235T mutation and essential hypertension [13]. Another study confirmed the association between M235T mutation and essential hypertension: a study on 63 white European families from the United Kingdom [4].

In another study conducted in the Japanese population of 352 individuals with a mean age of 52.5 years no association between M235T variant of the AGT gene and essential hypertension was found [17]. Rotimi *et al.* 1994, studied the

association between essential hypertension and M235T variant in the African -American population. They showed that the frequency of the T235 allele was 83% in hypertensive patients and 82% in control subjects. These results offered no evidence for a linkage between T235 allele and essential hypertension [18]. The study of Mettimano *et al.* (2001) showed that this polymorphism is very frequent in the Italian population sample. The frequency of homozygous patients for 235T allele is between 9 to 22% [19].

These variable results in different ethnic groups appear possibly because of the deficiencies in selection and definition of patients and controls regarding hypertension, family history, and also of the age of the first established diagnosis of essential hypertension. The results of our study suggest an association of the M235T polymorphism in the gene encoding angiotensinogen with essential hypertension. The TM genotype and the TT genotype were observed more frequently in hypertensive patients than in normotensive patients (52,63% heterozygotes in hypertensive group compared with 47,61% heterozygotes in the control group; 28,94% homozygotes in hypertensive group compare with 19,04% homozygotes in the control group). The frequency of carriers of M235T mutation was twice as high in the Romanian hypertensive population (81,57%) as in the Caucasian population (35-40%) [1].

The corroboration of these results suggests the interpretation that T allele of angiotensinogen M235T polymorphism is associated with inherited predisposition to essential hypertension in the Romanian population.

In conclusion we can say that there is a possible correlation between M235T polymorphism of the angiotensinogen gene and essential hypertension in Romanian patients. Patients heterozygous or homozygous for M235T mutation have an inherited predisposition to essential hypertension.

This is a preliminary study and more cases require further study to corroborate these observation. The 2 study groups were too small to carry out statistical analysis.

Because essential hypertension is a risk factor for coronary heart disease (CHD) or myocardial infarction (MI) (studies with sufficient sample size demonstrated a 2,6- fold increase for CHD and a 3,4- fold risk increase for MI) [20], we recommend that all patients with this abnormalities should be tested for M235T polymorphism. We also recommend that family members who have an index patient diagnosed with essential hypertension to be tested for this mutation in the angiotensinogen gene. The results will be more useful for the clinician since it seems that patients with one or two copies of T235 allele can present a greater decrease in blood pressure in response to ACE- inhibitory therapy compared to individuals with no copies of the T235 allele; patients with two copies of T235 allele respond to sodium reduction or weight reduction more likely than individuals with no copies of the T235 allele.

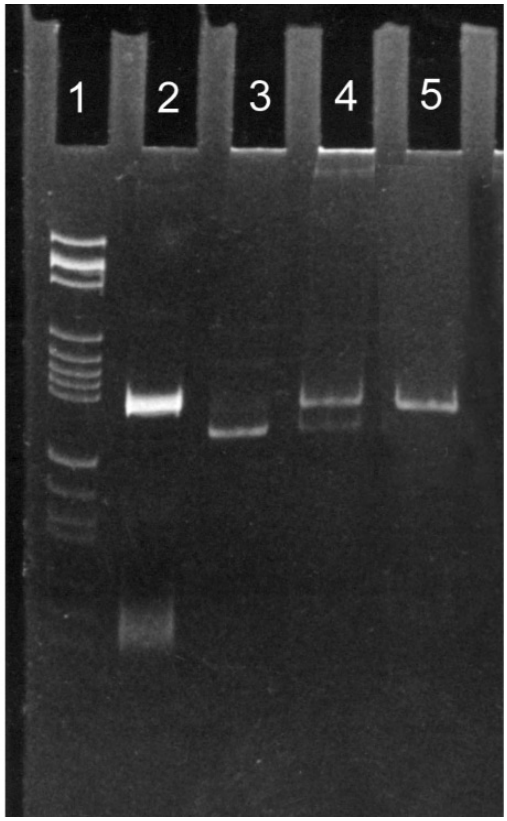

Fig. 1 Analysis of M235T DNA polymorphism in the angiotensinogen gene by 12% polyacrilamide gel electrophoresis. Lane 1: pBR322 Hae III digest (Sigma)- DNA molecular marker. Lane 2: 165bp amplified fragment. Lane 3: homozygous patient for M235T mutation: fragment of 141bp. Lane 4: heterozygous patient for M235T mutation: fragment of 165 and 141bp. Lane 5: negative patient for M235T mutation: fragments of 165.

References

1. **Jeunemaitre X., Soubrier F., Kotelevtsev Y.V., Lifton R. P., Williams C. S., Charru A., Hunt S.R., Hopkins P.N., Williams R.R., Lalouel JM., Corvol P.**, Molecular basis of human hypertension: role of angiotensinogen, *Cell*, **71**: 169-180, 1992
2. **Horan M., Lenfant C.,** Epidemiology of blood pressure and predictors of hypertension. *Hypertension*, **15**: 120-124, 1990
3. **Ward R.**, Familial aggregation and genetic epidemiology of blood pressure, In: Hypertension, Laragh J.H., Brenner B.M. eds., Pathophysiology, Diagnosis and Management, Raven Press, New York, 1990 pp. 81-100
4. **Caulfield M., Lavender P., Farrall M., Munroe P., Lawson M., Turner P., Clark A.J.,** Linkage of the angiotensinogen gene to essential hypertension, *N. Engl. J. Med.*, **330**: 1629-1633, 1994
5. **Harrap S.B.**, Cardiovascular disease and genetics of the renin- angiotensin system. *Am. Heart J.,***76**: 13-17, 1996
6. **Malik F.S., Lavie C.J., Mehra M.R.**, Milani R.V., **Re R.N.**, Renin- angiotensin system: genes to bedside, *Am. Heart J.*, **134**: 514-526, 1997
7. **Kageyama R., Ohkubo H., Nakanishi S.,** Primary structure of human preangiotensinogen deduced from the cloned DNA sequence, *Biochemistry,* **23**: 3603-3609, 1984
8. **Gaillard Sanchez I., Mattei M.G., Clauser E.,Carvol P.,** Assignment by in situ hybridization of angiotensinogen to chromosome band 1q4: the same region as the human renin gene, *Hum. Genet.,* **84**: 341-3, 1990
9. **Walker W.G., Whelton P.K., Saito H.,Russel R.P., Hermann J.,** Relation between blood pressure and renin, renin substrate, angiotensin II, aldosterone and urinary sodium and potassium in 574 ambulatory subjects, *Hypertension,* **1**: 287-9, 1979
10. **Fasola A.F., Martz B.L., Helmer O.M.**, Plasma renin activity during supine exercise in offspring of hypertensive parents, *J..Appl. Physiol*., **25**: 410, 1968
11. **Menard J., El Amrani A., Savoie F., Bouhnik J.,** Angiotensinogen: an attractive and underrated participant in hypertension and inflammation, *Hypertension,* **18**: 705, 770-776, 1991
12. **Jeunemaitre X., Inoue I., Williams C., Charru A., Tichet J., Powers M., Sharma A.M., Gimenez-Roqueplo A-P., Hata A., Corvol P., Lalouel J-M**., Haplotypes of angiotensinogen in essential hypertension., *Am. J. Hum. Genet.*, **60**: 1448-1460, 1997
13. **Jeunemaitre X., Charru A., Chatellier G.,** M235T variant of the human angiotensinogen gene in unselected hypertensive patients, *J. Hypertens. 11,* : S80-1, 1993
14. **Lahiri D. K., Nurnberger J. I. Jr.**, A rapid non-enzymatic method for the preparation of HMW DNA from blood for RFLP studies, *Nucleic Acids Res.*, **19**: 5444, 1991
15. **Russ A.P., Maerz W, Ruzicka V., Stein U., Gross W.,** Rapid detection of the hypertension- associated Met 235 Thr allele of the human angiotensinogen gene, *Hum. Mol. Genetics*, **2:** 609-610, 1993
16. **Niu T., Xu X., Rogus J., Zhou Y., Chen C., Yang J., Fang Z., Schmitz C., Zhao J., Rao S., Lindpaintner K.,** Angiotensinogen gene and hypertension in Chinese, *J. Clin. Invest*, **101:** 188-194, 1998
17. **Ischihara S., Yokota M., Fujimura T.**, Lack of association between variants of the angiotensinogen gene and the risk of coronary artery disease in middle-aged Japanese men, *Am. Heart J.*, **134:** 260-265, 1997
18. **Rotimi C., Morrison L., Cooper R., Oyejide C., Effiong E., Ladipo M., Osotemihen B., Ward R.,** Angiotensinogen gene in human hypertension. Lack of an association of the 235T allele among African Americans, *Hypertension*, **24:** 591-594, 1994
19. **Mettimano M., Lanni A., Migneco A., Specchia ML., Romano- Spica V., Savi L.,** Angiotensin-related genes involved in essential hypertension: allelic distribution in an Italian population sample, *Ital. Heart J*., **2:** 589-593, 2001
20. **Bloem L.J., Manatunga A.K., Tewksbury D.A., Pratt J.H.**, The serum angiotensinogen concentration and variants of the angiotensinogen gene in white and black children, *J. Clin. Invest.*, **95**: 948- 953, 1995